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Award Number: W81XWH-12-1-0014

TITLE: Regulation of ATM-Dependent DNA Damage Responses in Breast Cancer by the RhoGEF Net1

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REPORT DATE: April 2013

TYPE OF REPORT: Annual summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
April 2013	Annual summary	01-April-2012 – 31- March-2013

4. TITLE AND SUBTITLE
Regulation of ATM-Dependent DNA Damage Responses in Breast Cancer by the RhoGEF Net1

5a. CONTRACT NUMBER
W81XWH-12-1-0014

5b. GRANT NUMBER
W81XWH-12-1-0014

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Health Science Center

Houston, TX77030-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Neuroepithelioma transforming gene 1 (Net1) is a RhoA specific guanine nucleotide exchange factor (GEF) that is frequently overexpressed in human cancer, including breast cancer. We have previously reported that DNA damage activates Net1 to control RhoA and p38 MAPK mediated cell survival pathway in response to ionizing radiation (IR). However, others have shown that Net1 activation contributes to RhoB-mediated cell death after IR. Thus, the role of Net1 in controlling IR responses and cell survival is controversial. With the completion of the first year of this fellowship, we have found that the Net1A isoform is specifically required for DNA double-strand break (DSB)-induced signaling and DNA repair. Depletion of Net1A in human breast cancer cells reduced IR-stimulated ATM activation and signaling to its substrates Chk2 and H2AX. In addition, suppression of Net1A expression adversely affected cell survival after IR. Moreover, we observed that overexpression of the Net1A isoform significantly reduced γH2AX foci formation after IR, which required the unique N-terminal region of Net1A. Importantly, this effect did not require Rho GTPase activation by Net1A, and was not recapitulated by overexpression of RhoA or RhoB. Net1A was also found to co-immunoprecipitate with the DNA-PK complex in an IR-regulated manner. Taken together, our current data suggests a model in which Net1A functions as a non-catalytic binding protein to control DNA damage response signaling and DNA repair to affect cell survival after IR.

15. SUBJECT TERMS
Net1, RhoGEF, Ionizing radiation, DNA damage response, DNA repair

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	12	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

In the United States, breast cancer is the second most frequently diagnosed cancer in women (1). Ionizing radiation (IR) is commonly used to treat breast cancer patients after surgery, as well as for care of inoperable metastatic tumors (2). However, despite advances in the use of radiation therapy, a significant percentage of patients experience relapse of their cancers. Exposure to ionizing radiation (IR) causes double strand DNA breaks that are lethal to a cell if not repaired. In response to IR exposure a signal transduction cascade is initiated that activates cell cycle checkpoints to cause cell cycle arrest, thereby allowing the cells time to repair their damaged DNA (17). The ability of a cell to efficiently respond to IR treatment and evade cell death forms the basis for radiation resistance in cancer cells to allow for tumor recurrence. Thus, understanding the mechanisms controlling DNA damage signaling is necessary to devise more efficient treatments for breast cancer.

Rho GTPases control diverse aspects of cell behavior, including organization of the actin cytoskeleton, cell migration, cell cycle progression, and gene expression (4). Rho GTPases acts as molecular switches by cycling between an active GTP-bound and an inactive-GDP bound state (5). The activation of Rho GTPases is regulated by specific guanine nucleotide exchange factors (GEFs) (6). Net1 (Neuroepithelial transforming gene 1) is a RhoA subfamily specific GEF that was originally identified as a transforming gene in NIH3T3 cell focus formation assays (7). Upregulation of Rho protein activity has been shown to contribute to tumor initiation and progression, and aberrant regulation of Rho proteins has been found in tumor cells (8, 9). Recently, we have shown that Net1 is overexpressed in human breast carcinomas and its co-expression with the α6β4 integrin is associated with a high risk for distant metastasis in node positive breast cancer patients (10, 11, 12, 13). Previous work in our lab demonstrated that expression of the RhoA activating protein Net1 is required for cell survival following exposure to IR (3). Importantly, depletion of Net1 expression suppressed RhoA activation and induced cell death upon DNA damage (3). These results suggest that Net1 plays a critical role in the cellular response to DNA damage, and indicate that Net1 may be an attractive therapeutic target for sensitization of breast cancer cells to IR. Therefore, we decided to investigate the role of Net1 in DNA damage response signaling in breast cancer.

Body

Task 1: To determine the association of Net1 in DNA damage response signaling pathway (Months 1-12).

Task 1a: Determine the requirement for Net1 in activation of ATM, DNA-PK, and ATR in breast cancer cells by IR. To identify molecular mechanism by which Net1 affects DNA damage responses, we first tested whether Net1 is required for activation of checkpoint kinases. ATM is a central and primary signaling protein in the double strand DNA damage response (19). It is one of PIKKs, which include ATM, DNA-PK, and ATR. Like ATM, DNK-PK is also primarily activated upon DNA damage generated by IR. DNK-PK is a nuclear serine/threonine kinase which is composed of a catalytic subunit, DNK-PKcs, and a Ku70/80 heterodimer (22, 23). DNK-PK is also involved in DNA repair and damage checkpoint pathways (18). ATR responds to both UV and DSBs and shares several substrates with ATM, but is generally thought to play a minor role in the IR response. ATM phosphorylates many downstream targets to control DNA damage signaling, including Chk2 and p53 (20). This activation leads to apoptosis and transient cell cycle arrest. At the same time, activated ATM phosphorylates a histone protein variant called H2AX (18). In our previous work, we have shown that Net1, which is activated by IR, is required for cancer cell survival in response to DNA damage caused by CDT intoxication and IR (3). Another group also has shown that Net1 is activated after IR treatment (3). These studies suggest that Net1 may play an important role in the cellular response to DNA damage. We used MCF7 cells, which are a human breast cancer cell line that contains wild type p53 and responds well to IR. Cells were transfected with a control siRNA or an siRNA specific for both Net1 isoforms and then treated a low dose of IR. Phosphorylation of ATM and its downstream targets Chk2 and H2AX on their activating sites at various times after IR treatment was monitored by Western blotting. We observed that knockdown of both Net1 isoforms

attenuates the phosphorylation of ATM after IR treatment. Furthermore, reduced ATM activation in the Net1 knockdown cells was associated with decreased phosphorylation of the ATM substrates Chk2 and H2AX. We suggest that Net1 expression is required for efficient ATM, Chk2 and H2AX activation following IR (Figure 1).

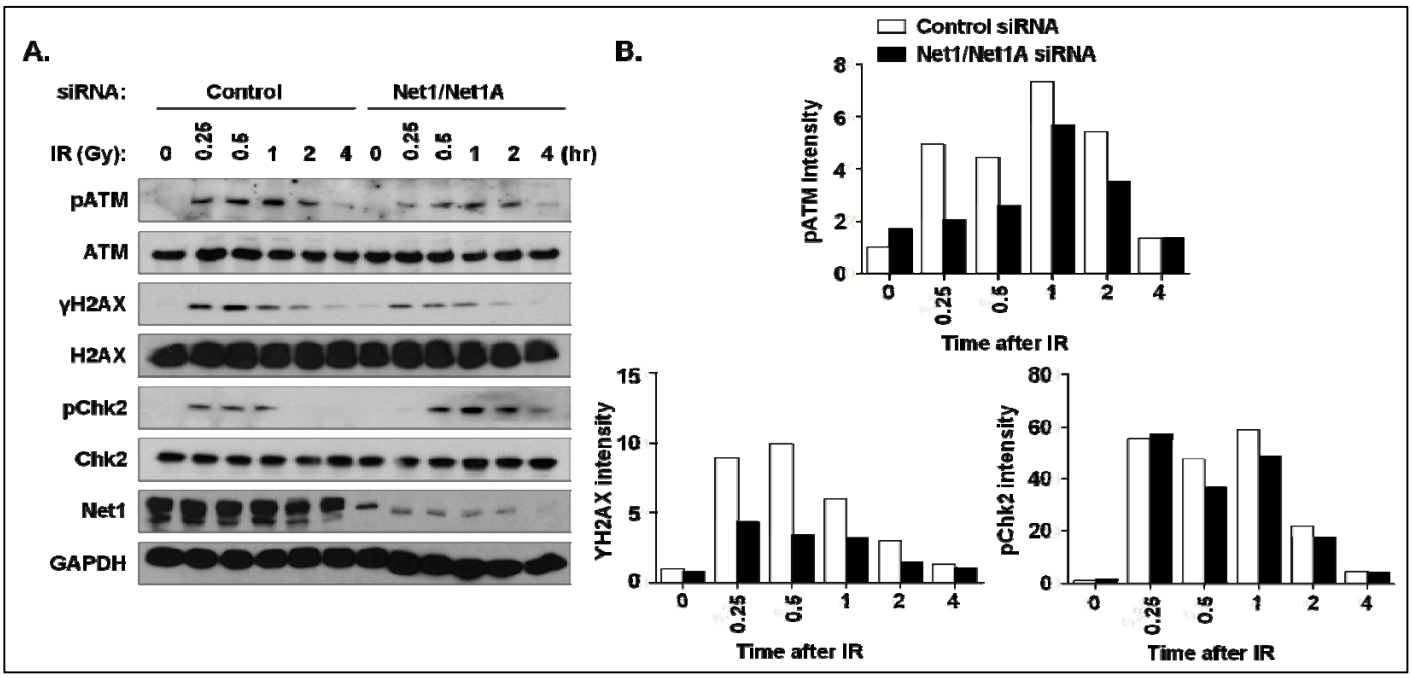

Figure1. Net1 is required for ATM-dependent DNA damage signaling. (A) MCF7 cells were transfected with control siRNA or an siRNA specific for both Net1 isoforms. Three days later, the cells were treated with 4-Gy IR and harvested at the indicated time points. Whole cell lysates were immunoblotted for the denoted antigens. Shown is a representative experiment from 3 independent experiments. (B) Quantification of phosphorylation of ATM and its substrates upon Net1 knockdown. Phosphorylation was normalized to total ATM, Chk2, and H2AX.

Two isoforms of Net1 exist in most cells, Net1 and Net1A. Both isoforms are predominantly localized in the nucleus, although Net1A lacks two of the nuclear localization signal (NLS) sequences present in Net1 and accumulates outside the nucleus when highly overexpressed (14). Nuclear localization of Net1 isoforms is an important negative regulatory mechanism to prevent Net1 dependent RhoA activation. This is illustrated by the findings that relocalization of Net1 outside the nucleus, either through N-terminal truncation or addition of a nuclear export signal sequence, results in highly elevated RhoA activation (16, 25). Importantly, deletion of the Net1 N-terminus creates a protein that is transforming in cultured cells (15, 27). Thus, identification of Net1 isoforms controlling DNA damage signaling is crucial to understanding their function in the DNA damage response. To determine which Net1 isoform is important for ATM-dependent DNA damage signaling, we transfected MCF7 cells with control or Net1 isoform specific siRNAs. We examined phosphorylation of ATM and its downstream signaling with different amounts IR. We observed that knockdown of Net1A dramatically decreased activation of ATM and its downstream targets Chk2 and H2AX following IR treatment. However, knockdown of the Net1 isoform was largely without effect (Figure 2). These results indicate that Net1A, but not Net1, is critical for the ATM-dependent DNA damage response following IR exposure in breast cancer cells.

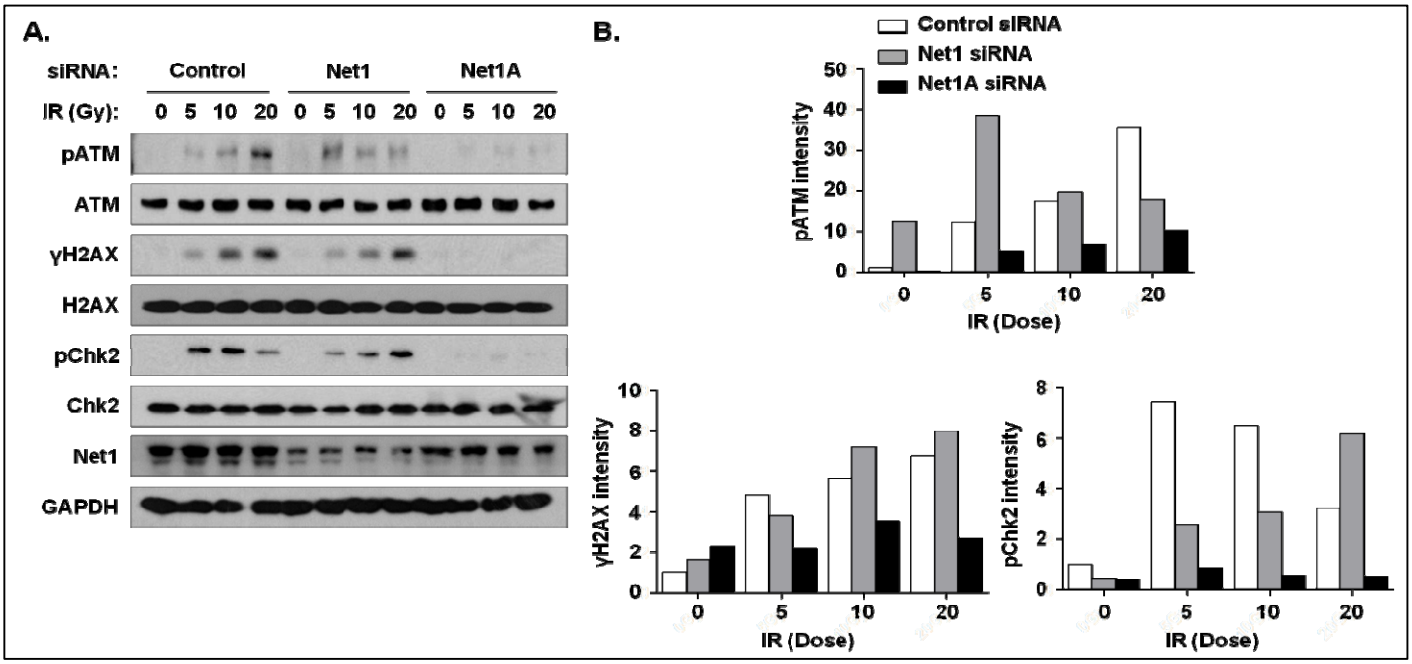

Figure 2. Knockdown of Net1A, but not Net1, reduces ATM activation after DNA damage. (A) MCF7 cells were transfected with control or Net1 isoform specific siRNAs. The cells were then treated with different amounts IR and harvested after 30 minutes. Phosphorylation or expression of the denoted proteins was detected by Western blotting. Shown is a representative experiment from 2 independent experiments. (B) Quantification of phosphorylation of ATM and its substrates in Net1 knockdown cells. Phosphorylation was normalized to total ATM, Chk2, and H2AX.

We initially proposed to establish *in vitro* kinase assay to test ATM/DNA-PK/ATR activation. However, due to problems with purification of proteins, we were unable to detect kinase activity. We will continue to establish protocols for measuring ATM and DNA-PK kinase activity in the next year. Since we detected the interaction of DNA-PK with Net1A (Figure 4), we expect that Net1A knockdown may augment DNA-PK kinase activity in MCF7 cells by IR treatment.

Task 1b: Determine the recruitment of PIKKs to IR-induced foci (IRIF) with Net1.

We clearly showed that Net1A knockdown reduces phosphorylation of ATM on its activating site in response to IR (Figures 1 and 2). The production of double strand breaks (DSBs) after IR treatment causes phosphorylation of H2AX, a variant form of histone H2A (17). H2AX phosphorylation is the major signal for recruitment of DNA damage response proteins and is mediated by the PIKK family. Phosphorylated H2AX, called γH2AX, localizes to sites of DNA damage called IR-induced foci (IRIF), which can be detected by indirect immunofluorescence. Within the PIKK family, ATM and DNA-PK phosphorylate H2AX in response to DSBs. To investigate whether Net1 controls the recruitment of PIKKs to IRIF, we examined whether siRNA mediated knockdown and overexpression of Net1A affects PIKK of γH2AX localization to IRIFs by immunofluorescence analysis. We measured the levels of pATM or γH2AX IRIF formation in IR-treated cells by analysis of photomicrographs using Image J software. We first determined whether overexpression of Net1A affects the recruitment of PIKKs to IRIFs, and checked for the co-localization of transfected Net1A with IRIFs. IR treatment induced a rapid increase of γH2AX and pATM in non-transfected MCF7 cells. In Net1A transfected cells we did not observe Net1A recruitment to IR-induced foci. Interestingly, we found that transient expression of Net1A strongly suppressed IR-induced foci formation in breast cancer cells after IR. Furthermore, expression of catalytically-inactive form of Net1A (L267E) also suppressed γH2AX staining, suggesting that

the ability of Net1A to activate RhoA or RhoB was not required. Similarly, expression of a constitutively active form of RhoA (L^{63}RhoA) did not affect IR-induced foci formation (Figure 3).

To further test whether Net1A might be required for IR-induced foci formation, we examined the ability of γH2AX and pATM to form foci in Net1A siRNA transfected cells after IR. However, we did not see a significant effect of Net1A knockdown on IRIF after IR in MCF7 cells. This may suggest compensation by the Net1 isoform in this assay (data not shown). In the future we will test the effect of knocking down both Net1 isoforms on IRIF staining, and rescue Net1 knockdown cells by expression of siRNA resistant forms of wild type Net1A, Net1, or different Net1/Net1A mutants.

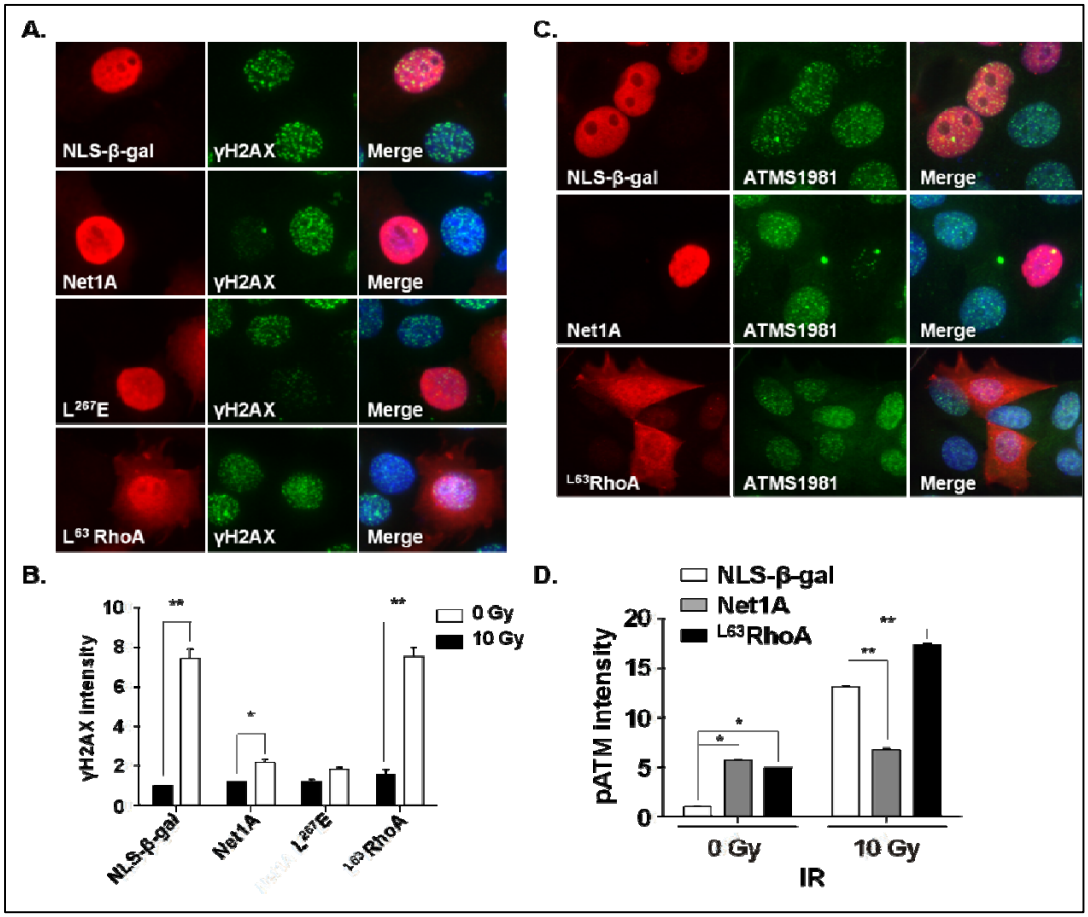

Figure 3. Net1A overexpression inhibits IR-induced DNA damage foci formation independent on its catalytic activity. (A) MCF7 cells were transfected with the control Myc-NLS-β-gal, HA-epitope tagged wild type Net1A, catalytically-inactive Net1A L^{267}E, or constitutively L^{63}RhoA. The cells were untreated or treated with 10-Gy IR, fixed and immunostained for γH2AX, HA and Myc antigens. Shown are IR-treated cells. (B) Quantification of γH2AX intensity from 3 independent experiments. Errors are SEM. * = $p<0.05$; ** = $p<0.01$. (C) MCF7 cells were transfected with Myc-NLS-β-gal, Myc-wild type Net1A, or constitutively active L^{63}RhoA. The cells were untreated or treated with 10-Gy IR, fixed and immunostained for pATM and Myc antigens. (D) Quantification of pATM intensity from single experiment. Errors are SEM. * = $p<0.05$; ** = $p<0.01$.

Task 1c: Identify the interaction of Net1 with PIKKs in response to IR.

In Task1a, Net1A knockdown attenuated the phosphorylation of ATM after IR treatment in breast cancer cells. To further characterize the regulation of DNA damage signaling by Net1A, we examined whether Net1A associates with ATM or DNA-PK in response to IR using a co-immunoprecipitation (coIP) assay. We found that Net1A did not interact with ATM in MCF7 cells in the presence or absence of IR (data not shown). We also tested whether Net1A associates with DNA-PK complex following IR. DNA-PK consists of a catalytic subunit (DNA-PKcs) and a DNA-end binding component, the Ku70/80 heterodimer. We observed that Net1 interacts with DNA-PK and Ku70/80, and this interaction was enhanced following IR treatment (Figure 4A). We confirmed DNA-PK interaction following Net1A overexpression in MCF7 cells (Figure 4B). We have begun to examine the domains within Net1A that are required for interaction with DNA-PK by expressing the N-terminal Net1A deletion mutant in MCF7 cells following IR treatment. We determined that the N-terminal domain of Net1A was required for with DNA-PK (Figure 4C). From these findings we conclude that Net1A interacts with the DNA-PK complex in an IR-regulated manner and that N-terminus of Net1A is required for this interaction.

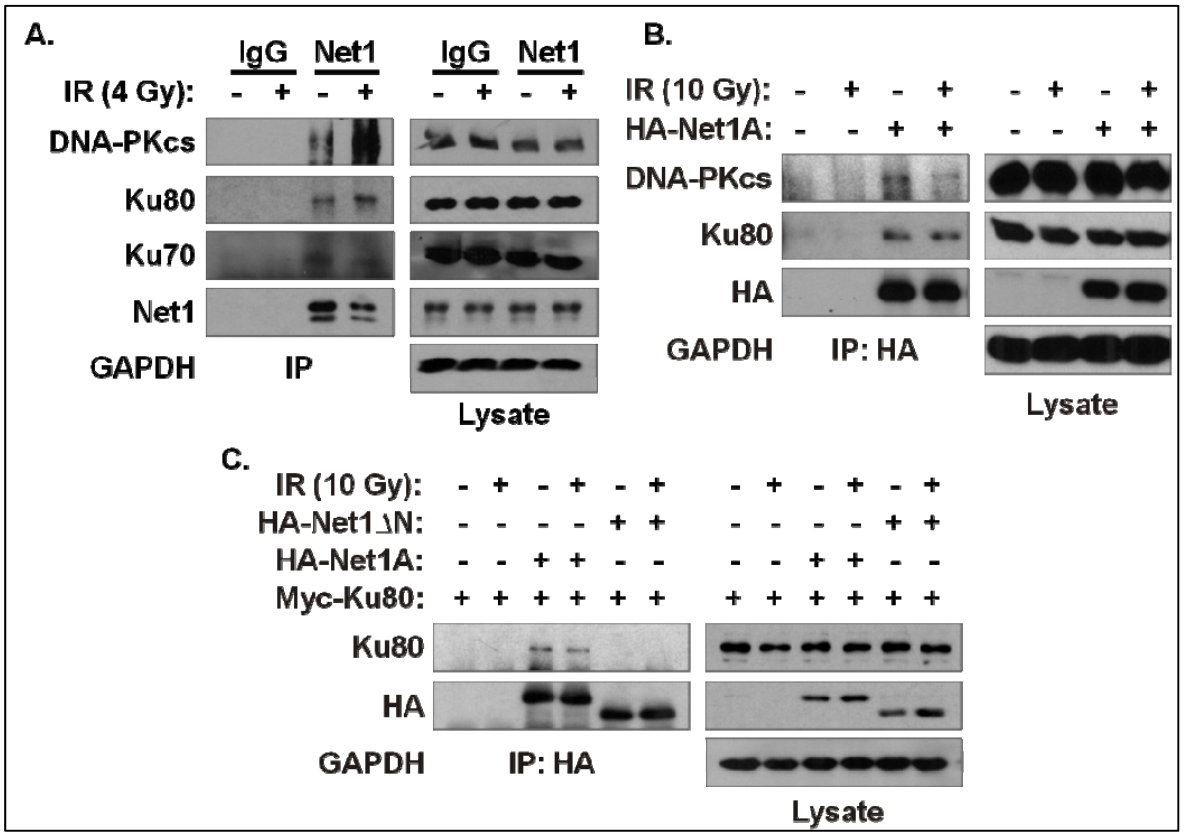

Figure 4. Net1A, but not Net1ΔN, co-immunoprecipitates with DNA-PK. (A) Endogenous Net1 interacts with the DNA-PK complex. MCF7 cells were treated 4-Gy IR. One hour after the cells were lysed and immunoprecipitations were performed using control IgG or Net1 antibodies. Co-precipitation of the indicated antigens was assessed by Western blotting. (B) Co-immunoprecipitation of HA-Net1A with DNA-PK complex. MCF7 cells were transfected with HA-epitope tagged Net1A and harvested 1h after IR. The lysates were immunoprecipitated with HA antibody and immunoblotted for the indicated antigens. (C) The N-terminus of Net1A is important for the interaction with the DNA-PK complex. MCF7 cells were transfected with HA-epitope tagged Net1A or the N-terminal deletion mutant Net1DN and harvested 1h after IR treatment. Lysates were immunoprecipitated with HA antibody and immunoblotted for the indicated antigens.

Task 2: To determine the physiological role of Net1 in cellular response to IR (Months 6-16).

Task 2a: Determine the role of Net1 in breast cancer cell survival in response to IR.

Our previous studies have shown that inhibition of Net1 expression sensitizes cells to the apoptotic effect of double strand DNA damage (3). Thus, this study indicates that Net1 regulates cell survival in response to double strand DNA damage. To determine whether this affects the long term viability of cells, we conducted clonogenic survival and MCA (Multicolor Competition Assay) (21) analysis following Net1 knockdown in MCF7 and MDA-MB-231 breast cancer cells, respectively. Clonogenic survival assays are the accepted standard for discerning effects on radiation sensitivity (26). MCA assays are useful for analyzing effects on cell growth for cell types that do not form coherent colonies. For clonogenic survival assays we used MCF7 cells, which are reflective of the luminal class of breast tumors that are often effectively treated by IR (24). For MCA assays we used MDA-MB-231 cells, which are representative of basal type tumors that are typically radiation resistant.

We first tested the functional importance of Net1A in cell survival following IR exposure in MCF7 cells. Cells were transfected with control or Net1A-specific siRNAs and then treated with different doses of IR. The cells were then plated and allowed to form colonies for two weeks. We observed that knockdown of Net1A reduced the number of colonies after IR (Figure 5). Therefore, this finding suggests that Net1A is required for MCF7 cell survival after exposure to IR.

For the MCA assay we generated MDA-MB-231 clones expressing either red fluorescence protein (RFP) or green fluorescence protein (GFP). We transfected cells with control siRNA (GFP expressors) or siRNA specific for both Net1 isoforms (RFP expressors), exposed to IR, and then let the cells grow for 7 days. We then determined the ratio of red to green cells using flow cytometry. Unfortunately, these assays were not informative. This is because the stable cell lines did not work properly when positive control proteins were knockdown down, such as ATM and Ku80 (data not shown). In future work we will reassess how to perform this assay.

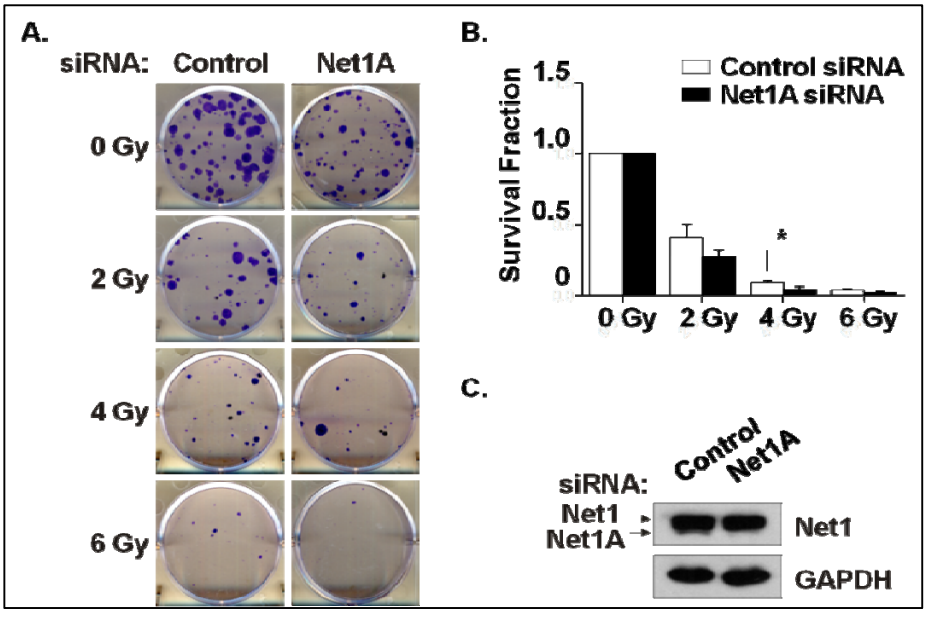

Figure 5. Net1A expression is required for cell survival after IR. (A) MCF7 cells were transfected with control or Net1A siRNAs. The cells were irradiated, replated and allowed to form colonies. (B) Quantification of survival fraction after IR. Shown is the mean of 3 independent experiments. Errors are SEM. * = $p<0.05$. (C) Representative Western blot of siRNA transfected cells.

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Key Research Accomplishments

- I determined that Net1A controls ATM activation after IR treatment.
- I determined that Net1A overexpression inhibits IR-induced DNA damage foci formation independent of its catalytic activity.
- I determined that Net1A interacts with the DNA-PK complex in response to IR.
- I determined that Net1A expression is required for MCF7 cell survival after DNA damage.

Reportable Outcomes

- My work was presented at the AACR annual meeting held in Washington, DC, USA, April 6-10, 2013. The abstract was entitled: Regulation of ATM-dependent DNA damage signaling in human breast cancer cells by the RhoGEF Net1A (Appendix 1).
- I also presented this work at a poster presentation at the annual University of Texas Health Science Center at Houston, Cell and Regulatory Biology Program Research retreat on April 11-12, 2013.

Conclusions

In our published studies Net1 expression is required for cell survival in response to DNA damage. However, there has been no molecular mechanism describing how Net1 regulates DNA damage responses. In the first year of this fellowship we showed that depletion of Net1 inhibited phosphorylation of ATM and its downstream substrates Chk2 and H2AX following IR exposure. This effect was specific for the Net1A isoform. Thus, a major finding of these studies is that Net1A plays an important role in controlling the activation of ATM in response to IR, as well as subsequent activation of Chk2 and H2AX. A second key finding of this study is that Net1A overexpression inhibits IR-induced DNA damage foci formation independent of its ability to stimulate the activity of its substrates RhoA or RhoB. This suggests that Net1A expression controls recruitment of PIKKs to IRIF after IR treatment. A third key finding of this study is that Net1A associates with the DNA-PK complex, which is a primary regulator of NHEJ type DNA repair after IR. Lastly, we demonstrated that Net1A depletion reduced cell survival after IR exposure. Together, these findings support a role for Net1A in DNA damage signaling and repair. Overall, the preliminary findings obtained during the first year of the current funding period will provide important knowledge on breast cancer research by identifying Net1A as a novel regulator of the double strand DNA damage response. The research conducted in this year will lead to the publication of a manuscript in the future. It may also serve as the basis for the discovery of new therapeutic strategies for breast cancer.

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Appendix

Appendix 1: Abstract from the 2013 American Association for Cancer Research (AACR) Annual Meeting and 2013 Annual Cancer Research Biochemistry Retreat

Regulation of ATM-dependent DNA damage signaling in human breast cancer cells by the RhoGEF Net1A

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The Neuroepithelioma transforming gene 1 (Net1) is a RhoGEF that is frequently overexpressed in human cancer, including breast cancer. We have previously reported that DNA damage activates Net1 to control p38 MAPK signaling and protect cells from ionizing radiation (IR) induced apoptosis. However, others have shown that Net1 activation contributes to cell death by activating a RhoB-Bim signaling pathway. Thus, the role of Net1 in controlling IR responses and cell survival is controversial. Here we show that the Net1A isoform is uniquely important for DNA double-strand break (DSB)-induced signaling and DNA repair. SiRNA-mediated knockdown of Net1A in human breast cancer cells reduced IR-stimulated ATM activation and signaling to its substrates Chk2 and H2AX. In addition, suppression of Net1A expression adversely affected cell survival after IR. We also observed that Net1A overexpression significantly reduced γH2AX foci formation after IR, which required the unique N-terminal region of Net1A. Importantly, this effect did not require Rho GTPase activation by Net1A, and was not recapitulated by overexpression of RhoA or RhoB. Using comet assays, we found that Net1A depletion substantially slowed DNA DSB repair in response to IR. Intriguingly, knockdown of Net1A promoted non-homologous end joining (NHEJ) in the absence of DNA damage, and Net1A was found to co-immunoprecipitate with the DNA-PK complex in an IR- regulated manner. Taken together, these data suggest a model in which Net1A functions as a non-catalytic binding protein to control DNA damage response signaling and DNA repair to affect cell survival after IR.